Macropexophagy in Hansenula polymorpha

Kiel, JAKW; Komduur, JA; van der Klei, IJ; Veenhuis, M

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Minireview

Macropexophagy in *Hansenula polymorpha*: facts and views

Jan A.K.W. Kiel, Janet A. Komduur, Ida J. van der Klei, Marten Veenhuis*

Eukaryotic Microbiology, Groningen Biomolecular Sciences and Biotechnology Institute (GBB), University of Groningen, Kerklaan 30, 9751 NN Haren, The Netherlands

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Abstract The hallmark of eukaryotic cells is compartmentalization of distinct cellular functions into specific organelles. This necessitates the cells to run energetically costly mechanisms to precisely control maintenance and function of these compartments. One of these continuously controls organelle activity and abundance, a process termed homeostasis. Yeast peroxisomes are favorable model systems for studies of organelle homeostasis because both the proliferation and degradation of these organelles can be readily manipulated. Here, we highlight recent achievements in regulation of peroxisome turnover in yeast, in particular *Hansenula polymorpha*, with a focus on directions of future research.

Key words: Methylotrophic yeast; Autophagy; Nitrogen limitation; Peroxisome; Peroxin

1. Introduction

Autophagic processes are predominantly considered a mode for the cell to remove wasted or redundant components. To a large extent this is indeed true, although also biosynthetic pathways are known that involve processes analogous to autophagy (Apg, e.g. the delivery of enzymes like aminopeptidase 1 to yeast vacuoles via the cytoplasm to vacuole targeting (Cvt) pathway, [1]). Apg ('self-eating'), as a cellular recycling mechanism, involves the function of the vacuole/lysosome, which supplies the hydrolytic enzymes required for the degradation process. Apg is a non-selective process that includes bulk turnover of portions of cytoplasm (including organelles). However, certain Apg-related processes are specific (e.g. turnover of redundant yeast peroxisomes). For a broad overview of Apg and related processes in various systems the reader is referred to Klionsky [2].

Yeast peroxisomes are ideal organelles for studies of autophagic processes because the proliferation, function and degradation of these organelles can easily be prescribed by manipulation of the growth conditions. This is in particular true for methylotrophic yeast species [3,4]. Peroxisomes are ubiquitous components of eukaryotic cells and are of unprecedented functional versatility [5,6]. Their importance is probably best illustrated by the existence of various inherited peroxisomal diseases in man (e.g. Zellweger syndrome) [7]. In fungi (including yeast) peroxisomes are predominantly involved in the metabolism of the carbon and/or nitrogen source used for growth, but may also serve biosynthetic functions (e.g. amino acid synthesis, synthesis of secondary metabolites such as β-lactams [8]).

This paper summarizes recent advancements in selective peroxisome degradation in methylotrophic yeast species, with focus on *Hansenula polymorpha*. Emphasis will be placed on challenging questions, namely (i) are specific subclasses of organelles protected from degradation, (ii) how do the mechanisms of peroxisome biogenesis and degradation interact and (iii) what is the origin of the membranes that sequester organelles destined for degradation?

2. Peroxisome biogenesis and selective degradation are interconnected processes in *H. polymorpha*

Peroxisome degradation in *H. polymorpha* can basically take place via two separate mechanisms, namely a general pathway (designated microautophagy: involving random turnover of cytoplasm) or a selective pathway (designated macropexophagy, involving the sequential selective degradation of individual peroxisomes). Microautophagy is characterized by random uptake of cytoplasm by the vacuole in a pinocytosis-like manner that does not involve previous sequestration. In *H. polymorpha*, this process is readily induced by limitation of the nitrogen source [9]. Macropexophagy is initiated when peroxisomes become redundant for growth [10] or become functionally inactive. Examples of the latter are observed in cells in which the peroxisomal matrix protein alcohol oxidase (AO) is chemically inactivated [11] or in which the peroxisomal membrane has been damaged by specific drugs [12].

In the related yeast *Pichia pastoris* the mode of peroxisome degradation depends on the carbon source used to induce the degradation process. Glucose addition induces a process designated micropexophagy, i.e. the bulk turnover of peroxisomes in the vacuole, whereas specific macropexophagy is observed when ethanol is used as inducer [13–15].

Micropexophagy is also observed in *Candida boidinii* [16,17]

*Corresponding author. Fax: (31)-50-3638280.
E-mail address: m.veenhuis@biol.rug.nl (M. Veenhuis).

Abbreviations: Cvt, cytoplasm to vacuole targeting; AO, alcohol oxidase; PDD, peroxisome degradation-deficient; Vps, vacuolar protein sorting; Apg, autophagy; End, endocytosis; Sc, *Saccharomyces cerevisiae*; Hp, *Hansenula polymorpha*; Pp, *Pichia pastoris*; PtdIns, phosphatidylinositol; Pex, peroxisome biogenesis; PAS, pre-autophagosomal structure; PE, phosphatidylethanolamine

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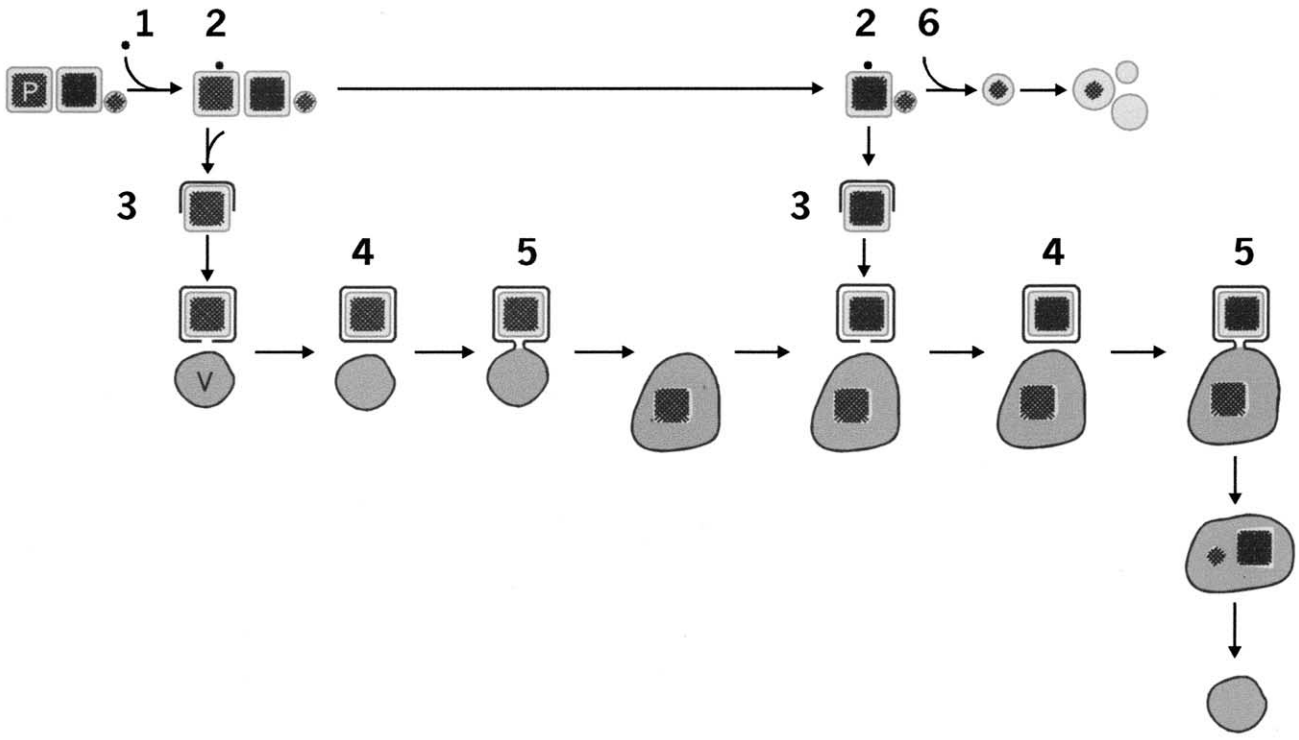

Fig. 1. Macropexophagy in *H. polymorpha*. Schematic representation of selective peroxisome degradation in *H. polymorpha*. After signalling (1), sequestration of the organelle tagged for degradation initiates at a focal spot on the peroxisomal membrane (2), followed by elongation of sequestration (3). Completion of sequestration requires a homotypic membrane fusion event to completely separate the organelle from the cytosol (4). Possibly, the spot of the homotypic fusion also specifies the spot of the fusion to the vacuole. Solely the outer membrane layer of the sequestered organelle fuses with the vacuolar membrane (a heterotypic fusion event; 5). After fusion, vacuolar hydrolytic enzymes degrade the organelle and its contents. Subsequently, more peroxisomes are sequestered and degraded. Finally, only one (or few) small, matrix protein import competent peroxisome(s) remain(s), that function(s) as the progenitor(s) of a new population of organelles upon renewed growth on media that require the function of the organelles (6). Key: P, peroxisome; V, vacuole.

but not in *H. polymorpha*. In the latter organism both glucose and ethanol induce specific macropexophagy. This indicates that species-dependent differences of peroxisome degradation in yeast do exist.

Peroxisome redundancy has been extensively studied in *H. polymorpha* cells that were shifted from methanol to fresh glucose-containing media. This glucose-induced carbon catabolite inactivation leads to a rapid destruction of the peroxisomal population that was present in the cells. Morphological analyses indicated that macropexophagy in *H. polymorpha* proceeds via sequential degradation of individual organelles and requires the vacuole (Fig. 1; see also below). These analyses also revealed that predominantly the large organelles of the cellular population are subject to degradation while one or few smaller organelles escape the degradation process [10,18] (Fig. 1). In line with this, invariably a low AO activity remains in cultures subjected to conditions that induce peroxisome degradation. The major question now is whether this protection to degradation is related to the capacity of the organelles to incorporate matrix proteins. This hypothesis is based on the following: peroxisome induction experiments have demonstrated that peroxisomes in wild-type *H. polymorpha* cells are sequentially formed. The most convincing example of this is observed after a shift of glucose-grown cells to fresh methanol media. Following the shift, the small organelles present in glucose-grown cells grow by the uptake of matrix proteins involved in methanol metabolism and, after maturation, multiply by division. We showed that, after fission, the mature organelles are no longer involved in the incorporation of bulk matrix proteins, and import is now confined to the newly formed organelles [18,19]. Upon induction of macropexophagy, invariably a single – or infrequently very few – organelle(s) escape(s) the degradation process [18]. It is tempting to suggest that these remaining peroxisomes are identical to those that display the enhanced matrix protein import capacity, a hypothesis that requires firm proof. However, recently we obtained the first experimental evidence consistent with the view that in *H. polymorpha* cells single immature organelles are indeed protected from glucose-induced degradation [20].

3. The specificity of macropexophagy

Various genes are now identified in *H. polymorpha* (designated *PDD*, peroxisome degradation-deficient) and *P. pastoris* that are required for selective and/or non-selective peroxisome degradation (reviewed in [21]). The general picture that emerges from their analysis is that many are homologues of baker's yeast genes that play essential roles in other vacuolar sorting routes as well. These include processes involved in the biogenesis of the vacuole [vacuolar protein sorting (Vps), Cvt] but also degradation processes [Apg, endocytosis (End)] [2,22]. Examples of such genes that have been identified in *H. polymorpha* are *PDD1* (homologue of *Saccharomyces cerevisiae VPS34p/END12*), *PDD7* (*ScAPG1/ScCVT10*), *PDD18* (*ScCVT9*) and *PDD19* (*ScVPS15*) ([23,24], our unpublished data). However, in *H. polymorpha* selective macropexophagy

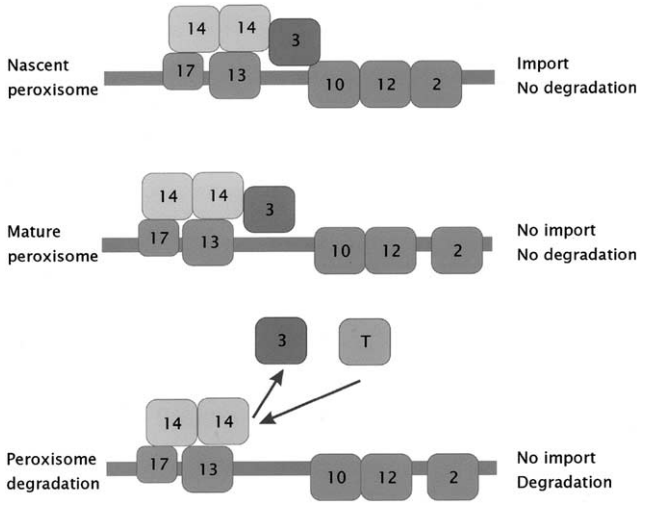

Fig. 2. Schematic representation of the putative function of *H. polymorpha* Pex3p and Pex14p in macropexophagy. Normal peroxisomes contain two membrane-bound protein complexes required for matrix protein import, namely a receptor-docking complex (Pex14p, Pex13p and Pex17p) and a RING finger complex (Pex2p, Pex10p and Pex12p) [32]. These complexes are connected by the peroxin Pex3p [33] thus allowing active matrix protein import. We speculate that organelle maturation is associated with separation of the two complexes, a process that is associated with the dissociation of Pex2p from the RING finger complex, rendering the organelle matrix protein import incompetent. In this view, Pex3p remains attached to the docking complex and may be involved in shielding Pex14p from recognition by a pre-existing protein moiety, designated terminator (T), that is required to initiate selective peroxisome degradation. In this model, induction of macropexophagy activates the removal and subsequent degradation (by the proteasome) of Pex3p molecules. Macropexophagy is therefore suggested to be dependent on the separation of the two complexes, thus also explaining why import competent organelles escape degradation. As a result of Pex3p removal, the putative terminator moiety can recognize the exposed Pex14p molecules and trigger sequestration-initiation of the organelle after which it is targeted to the vacuole (V) for degradation.

does not seem to utilize entirely the same machinery as degradation via non-selective microautophagy. This is indicated by the fact that in our collection of *H. polymorpha pdd* mutants strains are present that are affected in one of these pathways (microautophagy or macropexophagy) but not in both (e.g. *pdd2* [9]). This challenges the idea that all transport processes to the vacuole require the same sequestration and fusion mechanisms.

An even more challenging question that arises is how the specificity of the degradation process is organized. Destruction of cellular components is a hazardous cellular process that should be organized in a way that prevents unwanted degradation. Our work in *H. polymorpha* has provided the first clues that selective peroxisome degradation is organized at the level of peroxins, i.e. proteins that are essential for peroxisome biogenesis [25,26]. This was an unexpected result, although data of van der Klei et al. [27] indicated that the peroxisomal membrane is the prime target for the initiation of selective peroxisome degradation. Bellu et al. [25] showed that Pex14p, a protein involved in docking of the PTS1 receptor to the organelle membrane, is also essential for macropexophagy. In cells that form peroxisomes in the absence of Pex14p, sequestration was prevented. Hence, Pex14p is most likely required in an initial stage of the degradation process and in fact may act as a molecular switch where organelle development and degradation converge. The region that controlled degradation appeared to reside in the extreme N-terminus of Pex14p [25]. Very recent data show that also a second peroxin, Pex3p, plays a role in selective peroxisome degradation in *H. polymorpha* [26]. Its role differs however from that of Pex14p, in that not the *presence* but rather the *absence* of Pex3p appears to be a prerequisite to initiate macropexophagy. A hypothetical model that describes the function of both peroxins in macropexophagy is presented in Fig. 2.

4. What is the origin of the membranes that sequester peroxisomes during macropexophagy?

The first morphologically discernible step in the process of macropexophagy is the sequestration of an individual peroxisome tagged for degradation by a number of membrane layers [10,18]. This sequestration is a relatively fast process that may be completed within a time span of 10 min after its onset. The origin of the sequestering membranes is still an enigma. Occasionally vesicles or strands of endoplasmic reticulum have been observed close to sequestering peroxisomes (our unpublished data); also, close contacts have infrequently been observed between sequestering membranes and mitochondria [21]. However, there is no proof for a function of either of these organelles in peroxisome sequestration.

Morphological analysis convincingly showed that the development of sequestering membranes initiates at one spot at the peroxisomal membrane surface and continues from that site until the whole organelle is completely surrounded (see Fig. 1). What determines the start site of sequestration? The requirement of the peroxisomal membrane proteins Pex3p and Pex14p at the early stages of macropexophagy in *H. polymorpha* suggests a role for these proteins in the initial recognition process. Recent data indicate that both Pex3p and Pex14p are concentrated on spots at the peroxisomal membrane ([28]; our unpublished data) that may very well determine the sequestration-initiation site. In addition to this, formation of phosphatidylinositol (PtdIns) 3-phosphate by the lipid kinase Vps34p/Vps15p (in *H. polymorpha* Pdd1p/Pdd19p) is also required for the onset of macropexophagy ([23]; our unpublished data), which could suggest that this second messenger may provide the signal that initiates the sequestration event. Finally, yeast peroxisomes have been shown to be connected to actin microfilaments, which is essential for inheritance of these organelles [29]. It can be envisaged that the peroxisome-cytoskeleton attachment site may function as the sequestration-initiation site during macropexophagy.

Elongation of the sequestering membranes seems to occur via a 'zipper' mechanism in that during sequestration both the peroxisomal membrane and the delimiting membrane layers remain tightly connected. This suggests the presence of still unidentified proteins that retain a tight fit between the different membrane layers. Unfortunately, our current understanding of the composition of the sequestering membranes is very limited. Moreover, freeze fracture studies have supported the view that actually very few integral proteins are present in these membranes. This is an expected result in view of the fact that solely the outer layer of these membranes serves a function in sequestration and the subsequent heterotypic fusion with the vacuolar membrane while the other layers become destroyed together with the organelle.

Upon completion of the sequestration process, homotypic

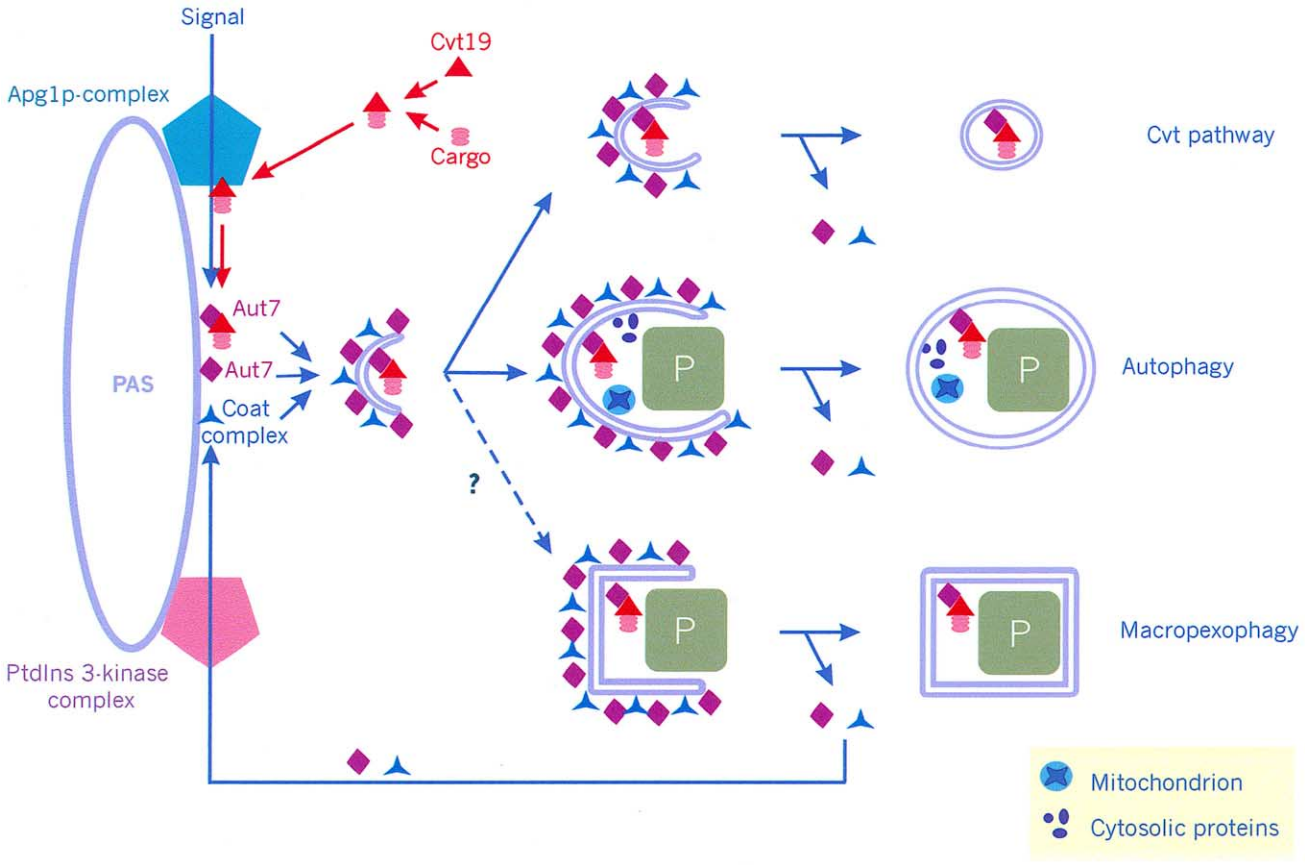

Fig. 3. Schematical model of sequestration of cargo molecules during Apg and the Cvt pathway in baker's yeast. Apg and Cvt processes require the function of the so-called pre-autophagosomal structure (PAS) that is probably the structure from which the membranes originate that sequester the respective cargo molecules (for review see [34]). Among the proteins present at the PAS are those of the so-called Apg1p-complex which is involved in the early stages of the Apg and Cvt routes, including ScApg1p, ScApg17p, ScApg13p, ScVac8p and ScCvt9p [31,35,36]. It is thought that the phosphorylation state of this complex regulates switching between the constitutive Cvt pathway and nitrogen limitation-induced Apg. At the PAS, the action of a PtdIns 3-kinase complex (consisting of ScVps34p, ScVps15p as well as ScV30p/Apg6p and ScApg14p [37]) recruits many proteins/complexes essential for both pathways. One of these includes a multimeric 350 kDa complex, consisting of ScApg5p, ScApg12p and ScApg16p [38] that, together, may form a network that constitutes a coat-like structure around newly forming autophagosomes and Cvt vesicles (designated 'coat complex' in the model). A key player at the PAS is ScAut7p. This protein is covalently bound to phosphatidylethanolamine (PE) and as such becomes incorporated into the PAS [39]. It can be envisaged that PE-associated ScAut7p drives the transport of membrane from the PAS, possibly in the form of vesicles, to newly forming autophagosomes or Cvt vesicles. Cargo selection in the constitutive Cvt pathway is thought to occur via a specific receptor (ScCvt19p [40]) that binds oligomeric cargo molecules (aminopeptidase 1 and α-mannosidase) and transports them to PAS-localized ScCvt9p. Here the ScCvt19p-cargo moieties are presumably passed on to PE-associated ScAut7p molecules that are incorporated into the growing Cvt vesicle. Upon completion, the vesicle releases its ScApg5p/ScApg12p/ScApg16p 'coat' as well as the ScAut7p molecules located on the outer surface of the Cvt vesicle, both of which are recycled to the PAS. This 'uncoating' presumably enables the vesicle to fuse to the vacuolar membrane. Together with the cargo, captured ScAut7p and ScCvt19p molecules become incorporated into the vacuole where they are degraded. Autophagosomes are thought to form in a similar manner. However, the larger size of autophagosomes necessitates insertion of enhanced amounts of phospholipids into these structures. Indeed, the level of ScAut7p is highly enhanced during Apg [41]. Moreover, Apg requires a functional flow of vesicles through the secretory pathway, suggesting that additional membrane material to build autophagosomes is derived from this route [42]. Whether cargo that becomes incorporated into autophagosomes is subjected to any kind of selection, is currently unknown. However, the receptor required for cargo selection in the Cvt pathway, ScCvt19p, is still required to insert aminopeptidase 1 and α-mannosidase into the growing autophagosome. We hypothesize that sequestration of peroxisomes during macropexophagy also requires the action of the PAS, and presumably also includes a role for Aut7p and the coat complex. Whether the cargo of the Cvt pathway also becomes included during sequestration is still an enigma.

membrane fusion events will finally produce a peroxisome that is completely separated from the cytosol, and which has now become competent to fuse to the vacuolar membrane. Completion of sequestration in the vicinity of the vacuole will allow for an efficient heterotypic fusion between the vacuolar membrane and the outer layer of the sequestering membranes. The specific t- and v-SNAREs that are required for these fusion events still remain to be identified. Additionally, it is yet unknown how the outer layer, after fusion with the vacuolar membrane, is protected from degradation by the vacuolar hydrolases that readily degrade the additional layers of the sequestering membranes.

As denoted above, certain genes involved in macropexophagy in *H. polymorpha* are also required for the morphologically highly similar Apg and Cvt pathways (reviewed in [21]). As both macropexophagy in *H. polymorpha* and the formation of autophagosomes and Cvt vesicles in baker's yeast require cargo sequestration, we have distilled a generalized model from the available data of the latter processes (Fig. 3). Can we use this model to obtain clues regarding seques-

tration of peroxisomes during macropexophagy? Clearly, certain proteins involved in the early steps of the Apg and Cvt pathways in baker's yeast (ScVps34p, ScVps15p, ScApg1p and ScCvt9p) have been found to be essential for the initiation of macropexophagy in *H. polymorpha* as well (Pdd1p, Pdd19p, Pdd7p and Pdd18p, respectively, [23,24], our unpublished data).

However, so far none of the proteins actually required for the formation of the Cvt vesicles or autophagosomes (e.g. ScApg5p, ScApg12p, ScApg16p) have been identified in screens for *H. polymorpha* mutants affected in macropexophagy. Furthermore, the *H. polymorpha pdd* mutants that are disturbed in the later stages of selective peroxisome degradation do not seem to be defective in microautophagy (e.g. *pdd2*, *pdd4* [9], our unpublished data). Nevertheless, recently the *P. pastoris* homologue of ScAut7p (PpPaz2p) was demonstrated to be required for macropexophagy [30]. However, a possible role in recruiting membranes to the sequestering peroxisome has so far not been considered for this protein.

In contrast to macropexophagy, the genes shown to be essential for micropexophagy in *P. pastoris* comprise not only those involved in the early steps in the Apg and Cvt pathways (homologues of ScVps15p, ScApg1p and ScCvt9p [30,31]), but also include those required for the cargo sequestration (homologues of ScAut7p and ScApg16p [30]). This suggests that micropexophagy utilizes key components of the Apg/Cvt sequestering machinery. Despite the absence of conclusive data we expect also an important function for the pre-autophagosomal structure (PAS) in macropexophagy, both during signalling as well as during organelle sequestration (Fig. 3). Whether attachment of the delimiting membranes to the peroxisome requires a ScAut7p homologue and whether these membranes are 'coated' with ScApg5p, ScApg12p and ScApg16p or distinct macropexophagy-specific proteins remains to be seen. Additionally, since selective degradation of peroxisomes occurs only upon its induction, we would predict that part of the specificity of the process is determined by a macropexophagy-specific receptor that, analogous to ScCvt19p, brings the organelle to the sequestering machinery (or vice versa). Such a receptor may very well represent the hypothetical terminator moiety (see Fig. 2), which is predicted to bind Pex14p at the peroxisomal membrane. Finally, we assume that the *H. polymorpha* PAS structure cannot form multiple Cvt vesicles or autophagosomes at the same time but does so consecutively. If this assumption were to be confirmed, it would also explain why macropexophagy involves sequential degradation of single peroxisomes rather than turnover of multiple organelles at the same time.

5. Concluding remarks

The methylotrophic yeast species *H. polymorpha* and *P. pastoris* have proved to be attractive model systems for the study of peroxisome homeostasis. In the near future, the availability of the complete genome sequences of these organisms will allow a genome-wide analysis of this process. Clearly, many intriguing questions remain. Among these are:

- What is the mechanism that ensures the peroxisomes are sequestered sequentially?
- What determines the specificity of peroxisome sequestration?
- What is the identity of the putative terminator moiety that is thought to initiate sequestration of peroxisomes?
- What is the precise role for the PAS in macropexophagy?
- Are homologues of ScAut7p and the autophagosome 'coat' proteins, ScApg5p, ScApg12p and ScApg16p, present on the membranes that sequester peroxisomes during macropexophagy?
- What is the protein content of sequestering membranes?
- How is the tight fit between the sequestering membranes and the peroxisomal membrane managed?

These and other questions should provide clues to understand whether macropexophagy is a mere variation of Apg or whether novel principles apply.

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